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In vitro antagonism of *Thielaviopsis paradoxa* by *Trichoderma longibrachiatum*

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Abstract

Seventy-nine *Trichoderma* strains were isolated from soil taken from 28 commercial plantations of *Agave tequilana* cv. 'Azul' in the State of Jalisco, Mexico. Nine of these isolates produced nonvolatile metabolites that completely inhibited the growth of *Thielaviopsis paradoxa* on potato dextrose agar plates. These isolates were identified as *Trichoderma longibrachiatum* on the basis of their morphology and DNA sequence analysis of two genes (ITS rDNA and translation elongation factor EF-1α). Mycoparasitism of *Th. paradoxa* by *T. longibrachiatum* strains in dual cultures was examined by scanning electron microscopy. The *Trichoderma* hyphae grew alongside the *Th. paradoxa* hyphae, but penetration of *Thielaviopsis* hyphae by *Trichoderma* was no apparent. Aleurioconidia of *Th. paradoxa* were parasitized by *Trichoderma*. Both hyphae and aleurioconidia of *Th. paradoxa* lost turgor pressure, wrinkled, collapsed and finally disintegrated. In liquid cultures, all nine *Trichoderma* isolates produced proteases, β-1,3-glucanases and chitinases that would be responsible for the degradation of *Thielaviopsis* hyphae. These results demonstrate that the modes of action of *T. longibrachiatum* involved against *Th. paradoxa in vitro* experiments are mycoparasitism and the production of nonvolatile toxic metabolites.

Key words: *Agave tequilana*, Antagonism, Mycoparasitism, *Thielaviopsis paradoxa*, *Trichoderma longibrachiatum*

Introduction

Thielaviopsis paradoxa is a soilborne plant pathogen that causes diseases in diverse economically important crop plants. This pathogen has been reported to attack date palm (*Phoenix dactylifera*) in the United States [1] and Kuwait [2]; pineapple (*Ananas comosus*) in Brazil [3]; and coconut (*Cocos nucifera*) in Venezuela [4]. Recently, *Th. paradoxa* has been found affecting *Agave tequilana* Weber cv. 'Azul' (Agave 'Azul') in Jalisco, Mexico, causing the wilt and death of >23% of these plants, where roots and stems are mainly attacked [5]. Agave 'Azul' is a very important Mexican crop used to produce the traditional beverage of Tequila by an alcoholic fermentation.

Traditionally, control of *Th. paradoxa* is achieved by cultural practices and fungicides such as benomyl and triadimefon [3]. However, it is desirable to integrate biological control agents into disease management plans to minimize the environmental impact of agrochemicals.

Trichoderma species are of agricultural importance because they have been used as agents for the biological control of phytopathogenic fungi, including soilborne, foliar and postharvest pathogens. *Trichoderma* species have been utilized to control root diseases caused by nematodes [6]; they may be also used to promote plant growth [7, 8] and to induce disease resistance in plants [8, 9]. The mechanisms by which *Trichoderma* species control plant pathogens include mycoparasitism [10, 11], competition for nutrients and growth space [12], and production of toxic metabolites, including the antibiotics [13, 14].

Effective biological control agents are often isolated from the root or rhizosphere of a specific crop where biocontrol activity is suspected to occur. Such 'rhizosphere competent' strains are better adapted to crop field conditions and may provide better control of diseases than those strains isolates from other sources [15]. The objectives of the present research were (i) to isolate *Trichoderma* strains from soil samples collected on Agave 'Azul' fields; and (ii) to investigate the mechanisms of biocontrol involved in any antagonistic activity against *Th. paradoxa*.

Materials and methods

Fungal strains

In February 2002, soil samples from twenty-eight commercial plantations of Agave 'Azul', were obtained from four localities of Jalisco State, Mexico (Acatic, Tepatitlán, Las Motas and Arandas). Five soil samples of approximately 1 kg each were collected from the rhizosphere (in a depth of 0–15 cm) of each plantation, placed into polyethylene bags and stored at 4°C. The isolation of *Trichoderma* strains was carried out by the dilution plating method proposed by Heller and Theiler-Hedtrich [16]. Five Petri dishes containing acidified V8 juice agar were inoculated with 0.1 ml of each diluted soil sample (1/1000, w/v) and then incubated for 3 days at 25°C. Spores of *Trichoderma* strains were finally transferred to Petri dishes, containing potato dextrose agar (PDA, Difco), to obtain pure cultures. The nine most antagonistic of these strains (Table 1) were deposited in the American Type Culture Collection (ATCC; Manassas, Virginia, USA) and the Centraalbureau voor Schimmelcultures (CBS; Utrecht, The Netherlands). The reference numbers for each strain are given in Table 1. The phytopathogen utilized in this study was *Th. paradoxa* (ATCC MYA-1387). All the fungal strains were maintained on PDA slants at 5°C.

Morphological observations

Isolates of *Trichoderma* were cultured on cornmeal dextrose agar (CMD; Difco cornmeal agar, 2% dextrose, 1% antibiotic solution [0.2% of Sigma streptomycin sulfate and 0.2% of Sigma neomycin sulfate]). Petri dishes (9 cm-diam) containing the above medium were incubated at 20°C with 12 h cool white fluorescent light and 12 h darkness for 1 week. Characteristic of colony appearance as the presence of diffusing pigment in the medium, the

Table 1. Trichoderma longibrachiatum strains isolated from the rhizosphere of *Agave tequilana* cv. 'Azul' cultures in different localities of Jalisco State, Mexico

Strain	ATCC[a] number	CBS[b] number	Locality	Geographic origin	GenBank accession number (ITS1, 5.8s & ITS2)	GenBank accession number (EF-1α)
V.S.L. 22	MYA-3646	118638	Acatic	LN 20° 43' 04.0'' LO 102° 55' 24.2''	DQ297054	DQ297064
V.S.L. 41	MYA-3647	118641	Acatic	LN 20° 43' 28.6'' LO 102° 53' 33.2''	DQ297055	DQ297067
V.S.L. 62	MYA-3643	118640	Tepatitlán	LN 20° 47' 50.3'' LO 102° 45' 22.7''	DQ297056	DQ297069
V.S.L. 65	MYA-3645	118643	Tepatitlán	LN 20° 47' 50.3'' LO 102° 45' 22.7''	DQ297057	DQ297062
V.S.L. 103	MYA-3651	118642	Tepatitlán	LN 20° 47' 25.5'' LO 102° 44' 37.3''	DQ297053	DQ297066
V.S.L. 131	MYA-3649	118637	Tepatitlán	LN 20° 46' 32.1'' LO 102° 44' 08.2''	DQ297058	DQ297063
V.S.L. 133	MYA-3648	118636	Tepatitlán	LN 20° 46' 32.1'' LO 102° 44' 08.2''	DQ297059	DQ297068
V.S.L. 152	MYA-3650	118644	Tepatitlán	LN 20° 46' 34.1'' LO 102° 43' 57.8''	DQ297060	DQ297065
V.S.L. 243	MYA-3644	118639	Arandas	LN 20° 40' 36.1'' LO 102° 40' 02.7''	DQ297061	DQ297070

[a] American Type Culture Collection, Manassas, Virginia, USA.
[b] Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

conidial colour and the odour were described and recorded. Microscopic observations were carried out from preparations initially mounted in 3% KOH followed by distilled water. Thirty measurements for each of the following structures were made for each isolate: conidium length and width, phialide length and width, base of phialide, cells supporting the phialides and chlamydospore length and width. All measurements were made from digital images (magnification of 1600) taken using the beta 4.0.2 version of Scion Image (Scion Corp., Frederick, MD, USA). *Trichoderma* strains were identified using the interactive key available at http://nt.ars-grin.gov/taxadescriptions/keys/TrichodermaIndex.cfm.

DNA extraction, amplification and sequencing

DNA from *Trichoderma* strains was extracted, according to previously described methods [17]. Two gene regions: internal transcribed spacers (ITS1, 5.8S, ITS2 rDNA), and translation elongation factor (EF-1α) were amplified by PCR using the primers ITS1 and ITS4 for ITS [18], and the primers EF1-728F [19] and TEF1 rev [20] for EF-1α. PCR was carried out using a PTC-200 thermocycler (MJ Research, Waltham, MA, USA), in a total volume of 25 μl containing 2.5 μl of 10 × Buffer (New England Biolabs, Ipswich, MA, USA), 0.5 μl of 10 mM dNTPs, 0.5 μl of 10 μM forward primer, 0.5 μl of 10 μM reverse primer, 0.25 μl of *Taq* Polymerase (New England Biolabs, Ipswich, MA, USA), 0.5 μl of 10–100 ng/μl genomic DNA and 20.25 μl of distilled water. The amplification program included an initial denaturalization at 95°C for 5 min, followed by 30 cycles of 30 s at 94°C, 30 s at 45°C, 1 min at 72°C, and a final extension of 10 min at 72°C. PCR products were purified with the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA, USA). 10 μl of DNA sequencing reactions were performed using the Big Dye Terminator Cycle Sequencing kit (Perkin Elmer Applied Biosystems, Foster City, CA, USA), according to the manufacturer's specifications, and the products were analyzed directly on ABI prism 3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Molecular analyses

DNA sequences were edited and assembled using the software Sequencher 4.2.2 (Gene Codes, Madison, WI, USA), aligned with Clustal X 1.81 and then visually adjusted [21]. The sequences were BLAST-searched against sequences available in GenBank. The sequences obtained during this study were deposited in the GenBank, and the accession numbers are given in Table 1.

Effect of diffusible, nonvolatile metabolites produced by Trichoderma strains on mycelial growth of Thielaviopsis paradoxa

The effect of diffusible, nonvolatile metabolites produced by *Trichoderma* isolates on the growth of *Th. paradoxa* was assessed using the cellophane method proposed by Dennis and Webster [22]. A sterile cellophane membrane (9 cm-diam) was placed on the surface of PDA in plates contained 25 ml of medium. A 5 mm mycelial plug from an actively growing edge of a 3 day-old colony of each isolate of *Trichoderma* was placed in the center of each cellophane membrane. The Petri dishes were incubated for 2 days at 25°C in darkness, after which the cellophane membrane with the fungal mycelia was removed. A 5 mm mycelial plug, taken from an actively growing edge of 3 days-old colony of *Th. paradoxa*, was placed on the center of the plate in the same position where a *Trichoderma* isolate was grown. The plates were incubated at 25°C in darkness for 4 days, and the diameters of each fungal colony were measured. Controls were prepared by placing a 5 mm mycelial plug of *Th. paradoxa* instead of the antagonist on the pre-inoculated *Thielaviopsis* plate. Each treatment was replicated four times.

Mycoparasitism

Mycoparasitism of *Th. paradoxa* by *T. longibrachiatum* strains was studied in dual cultures, following the procedure described by Dennis and Webster [23]. Petri dishes (9 cm-diam) containing 25 ml of PDA were inoculated with mycelial plugs (5 mm-diam) taken from an actively growing edge of 3 day-old colonies of both fungi. These mycelial disks were simultaneously placed 5 cm apart from

each other on the surface of the medium. The plates were incubated at 25°C under continuous light for 7 days. The time for the first contact between the antagonist and the pathogen, and the advance of antagonism on the pathogen colony were measured. Control plates were prepared by inoculating only *Th. paradoxa*. Each treatment was replicated four times.

Scanning electron microscopy

Mycelial samples (0.5 cm^2) were cut from the interaction zone between the antagonist and phytopathogen 2 days after the first contact was established in a dual culture. These samples were vapor-fixed with 2% (w/v) osmium tetroxide for 20 h at room temperature; air dried and kept in a desiccator [11]. The pieces were mounted on copper stubs, coated with gold-palladium using a sputter coater (JFC-1100), and examined with a scanning electron microscope (JEOL, JSM-35C) at 15 kV. One piece of each *T. longibrachiatum* strain paired with the pathogen was examined.

Characterization of extracellular enzymes

Production of extracellular enzymes by *T. longibrachiatum* strains was studied using the medium described by Anjani Kumari and Panda [24]. For chitinase production, 12.5 g of chitin and 2.8 g $(NH_4)_2SO_4$ were added as the sole carbon and nitrogen sources respectively, and glucose, citric acid, peptone and urea were eliminated from the medium [25]. The initial pH of the media was adjusted to 5.0 for the production of proteases [26] and β-1,3-glucanases [27], and to 5.6 for chitinase production [25]. Finally, these media were sterilized (121°C for 20 min). Erlenmeyer flasks (250 ml) containing 100 ml of medium were inoculated with 0.2 ml of a spore suspension (10^7 spores per ml) of each *T. longibrachiatum* strain from a 5 days-old culture on PDA plates. The flasks were incubated on a rotary shaker at 180 rpm and 30°C for 36 h for β-1,3-glucanases [27], at 28°C for 36 h for proteases and at 28°C for 43 h for chitinases [25]. After incubation, 5% (v/v) of mycelial suspension was used to inoculate the production media. Five ml of fermented liquid were removed daily from each flask for 4 days. Samples were then centrifuged at 3500 rpm for 10 min at 4°C and the supernatants were filtered with Whatman No. 1 filter paper and used to assay enzyme activities for proteases and chitinases [25], and β-1,3-glucanases [27]. Each assay consisted of four replicates. Single units of protease, β-1,3-glucanase and chitinase activity were defined as the amount of enzyme that catalyzed the released of 1 μmol of tyrosine, glucose and *N*-acetylglucosamine per minute, respectively. All cultures and enzyme assays were repeated independently with similar results.

Results

Effect of nonvolatile metabolites produced by Trichoderma strains on the Thielaviopsis paradoxa growth

A total of 79 *Trichoderma* strains were isolated from 28 soil samples collected from the rhizosphere of cultivated areas of Agave 'Azul' in Jalisco state, Mexico. The isolated strains were cultured on PDA covered by cellophane membranes. After 2 days of incubation, the membranes containing the biomass of each of the 79 *Trichoderma* strains were removed, and *Th. paradoxa* was then inoculated onto the semi-solid media in order to determinate the effect of nonvolatile metabolites produced by *Trichoderma* on its growth.

Only nine of those strains completely inhibited the mycelial growth of *Th. paradoxa* during the 12 days of the experiment. It was hypothesized that a diffusible non-volatile substance was responsible for the inhibiting of *Thielaviopsis* growth. These nine isolates were selected for further studies and were identified as *T. longibrachiatum* based on their morphological characteristics and DNA sequence analysis of two genes (ITS rDNA and translation elongation factor EF-1α) (Table 1).

Mycoparasitism

In order to study parasitism of the nine *T. longibrachiatum* strains on *Th. paradoxa*, dual cultures were established. Because the radial growth rates of the individual strains of *T. longibrachiatum* were different, the time of contact with the *Thielaviopsis* culture differed among the isolates (Table 2). However, the growth of *Th. paradoxa* stopped as soon as contact with the *Trichoderma* was

Table 2. Dual cultures of *Trichoderma longibrachiatum* and *Thielaviopsis paradoxa*

Trichoderma strains	Time of first contact with *Th. paradoxa* (h)	Overgrowth on colonies of *Th. paradoxa* (mm)		
		At 3 days	At 4 days	At 5 days
V.S.L. 41	51.80 (± 0.47)	4.75 (± 0.95)	7.25 (± 0.50)	11.00 (± 1.80)
V.S.L. 103	50.20 (± 0.50)	8.75 (± 0.95)	25.75 (± 2.21)	38.75 (± 2.21)
V.S.L. 22	41.00 (± 0.40)	12.75 (± 0.95)	24.75 (± 3.59)	30.50 (± 1.29)
V.S.L. 133	40.70 (± 0.28)	11.75 (± 2.06)	34.50 (± 1.00)	-
V.S.L. 65	40.60 (± 0.25)	7.75 (± 0.95)	28.50 (± 1.73)	37.00 (± 0.81)
V.S.L. 243	40.50 (± 0.40)	14.25 (± 0.95)	34.75 (± 0.50)	-
V.S.L. 62	39.50 (± 0.40)	13.25 (± 1.50)	35.75 (± 1.70)	-
V.S.L. 152	39.20 (± 0.28)	22.50 (± 1.73)	32.00 (± 1.82)	36.50 (± 1.29)
V.S.L. 131	38.50 (± 0.40)	15.50 (± 1.29)	30.75 (± 0.95)	36.75 (± 1.50)

All values are means ± SD of four replicates.

established. Generally, *T. longibrachiatum* rapidly overgrew the phytopathogen colony and a complete invasion and sporulation occurred after 5 days of culture (Table 2). One *Trichoderma* strain, V.S.L. 41, grew slower than the others and only after 7 days of incubation the invasion and sporulation on the *Thielaviopsis* colony was fully consummated. On the other hand, cultures of *Th. paradoxa* minus antagonist completely covered Petri dishes after 6 days.

Mycoparasitism observed by scanning electron microscopy

Two days after the first mycelial contact was established in dual cultures, observations of mycoparasitism were carried out by scanning electron microscopy (Figure 1). Hyphae of the *T. longibrachiatum* strains formed an appressorium-like structure (Figure 1a, b) in close contact with the aleurioconidial chains of *Th. paradoxa*. Isolates V.S.L. 62 and V.S.L. 133 produced penetration holes (not shown) in *Th. paradoxa* aleurioconidia. The penetrated aleurioconidia rapidly lost turgor and collapsed (Figure 1c, e). Interestingly, during the contact with the host aleurioconidia, hyphae of V.S.L. 41 strain branched dichotomously at the tip (Figure 1c).

The hyphae of *T. longibrachiatum* were not observed to coil around the hyphae of *Th. paradoxa*. Instead, the hyphae of *T. longibrachiatum* grew alongside the hyphae of *Th. paradoxa;* however, penetration was not evident (Figure 1d). Despite the absence of visible penetration, *Th. paradoxa* hyphae lost turgor pressure, wrinkled and collapsed (Figure 1e). Finally, both hyphae and aleurioconidia of *Th. paradoxa* completely disintegrated (Figure 1f).

Characterization of extracellular enzymes

The production of proteases, β-1,3-glucanases and chitinases by the nine isolates of *T. longibrachiatum* was studied in liquid culture. In general, all the *T. longibrachiatum* strains were able to produce the most important extracellular enzymes involved on the cell wall degradation of pathogenic fungi: proteases, β-1,3-glucanases and chitinases (Figure 2). The strains varied in the time at which they reached maximal production of each enzyme. In the case of proteases, for most of the antagonistic isolates, maximum enzymatic activity occurred at 48 h of culture (Figure 2a); however, this activity practically disappeared 24 h later. Remarkably, a maximum activity of 2.1 ± 0.15 U L^{-1} was obtained by V.S.L. 62.

β-1,3-glucanase activity was detected after 24 h of culture for all the strains (Figure 2b); however, maximal activities were obtained at different incubation times, according to the tested strain: V.S.L. 22, 76.1 ± 7.83 U L^{-1} at 24 h; V.S.L. 103, 89.2 ± 4.90 U L^{-1} at 48 h; V.S.L. 133, 72.0 ± 10.2 U L^{-1} at 72 h. The V.S.L. 22 strain was unusual in producing two peaks of activity, one at 24 h and one at 96 h (74.7 ± 4.54 U L^{-1}).

Chitinase activity was detected at 48 h of incubation for most of the strains and it was observed to continue from 48 to 96 h (Figure 2c). A maximum enzyme production was reached by V.S.L. 152 (0.3 ± 0.008 U L^{-1}) at 48 h. V.S.L. 22,

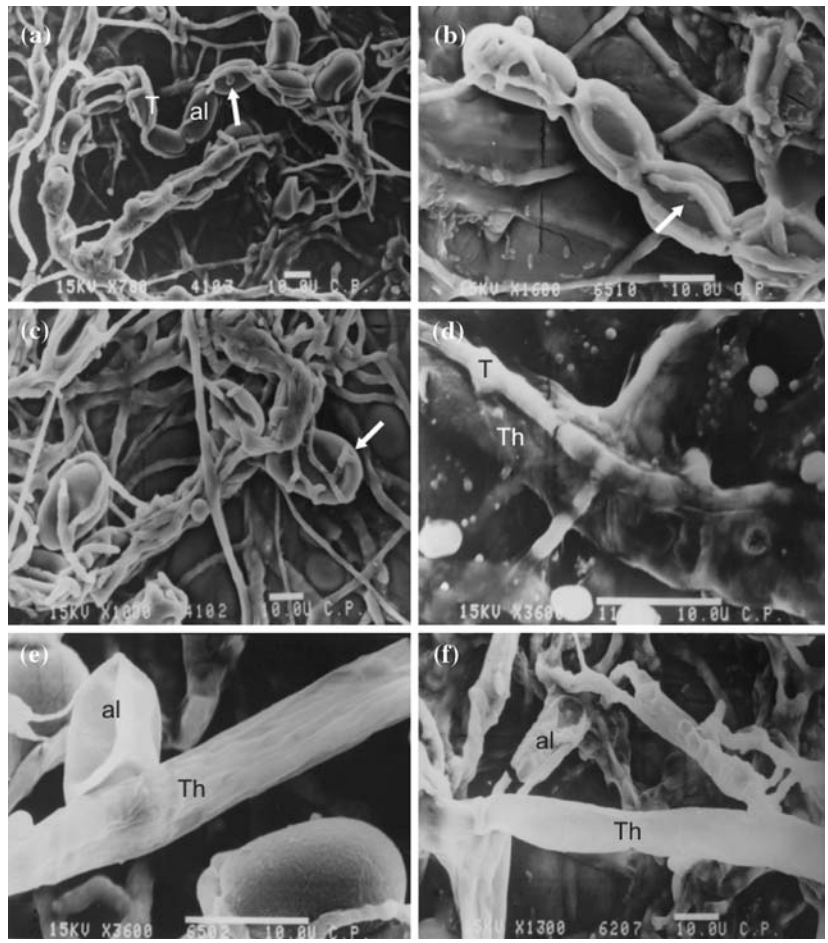

Figure 1. Scanning electron micrographs illustrating the mycoparasitism of *Trichoderma longibrachiatum* (T) on *Thielaviopsis paradoxa* (Th) 2 days after their first contact in a dual culture. (**a**) V.S.L. 41 hyphae adhering to aleurioconidia (al) chain of *Th. paradoxa*. (**b**) V.S.L. 65 hyphae parasitizing an aleurioconidia chain of *Th. paradoxa*. (**c**) Chain of aleurioconidia of *Th. paradoxa* wrapped by V.S.L. 41 hyphae and losing its turgor. Dichotomous tips of the antagonist hyphae are also visible (arrow). (**d**) A hypha of V.S.L. 131 growing along of a hypha of *Th. paradoxa*. (**e**) Hyphae and aleurioconidia (al) of *Th. paradoxa* collapsed by V.S.L. 65. (**f**) Hyphae and aleurioconidia (al) of *Th. paradoxa* completely disintegrated by V.S.L. 62. Arrows in (**a**) and (**b**) indicate the incipient formation of an appresorium-like structure on the aleurioconidia surface.

V.S.L. 41 and V.S.L. 65 strains did not secrete detectable levels of chitinases.

Discussion

It was not a surprise that *Trichoderma* strains were isolated from each of the 28 samples of soils collected from the rhizosphere of cultivated areas of Agave 'Azul' in Jalisco State, Mexico. However, it was a surprise that the predominant species recovered was *T. longibrachiatum*. Because this species is unusual in *Trichoderma* in being able to grow and sporulate at 40°C [28], and is also the most common species involved in human disease [29]. It is tempting to suggest that it is one of the few species adapted to survival in the exposed soils in which Agave 'Azul' is cultivated in Jalisco State. To our knowledge, this is the first report of the isolation of *Trichoderma* from the Agave ecosystem. *T. longibrachiatum* has been isolated in North and South America, Europe, Africa and India [28].

Species of *Trichoderma* are known to produce nonvolatile metabolites, such as antibiotics [30] and enzymes [26, 31, 32] that are involved on the inhibition of growth of phytopathogenic fungi. Among the 79 *Trichoderma* strains that we

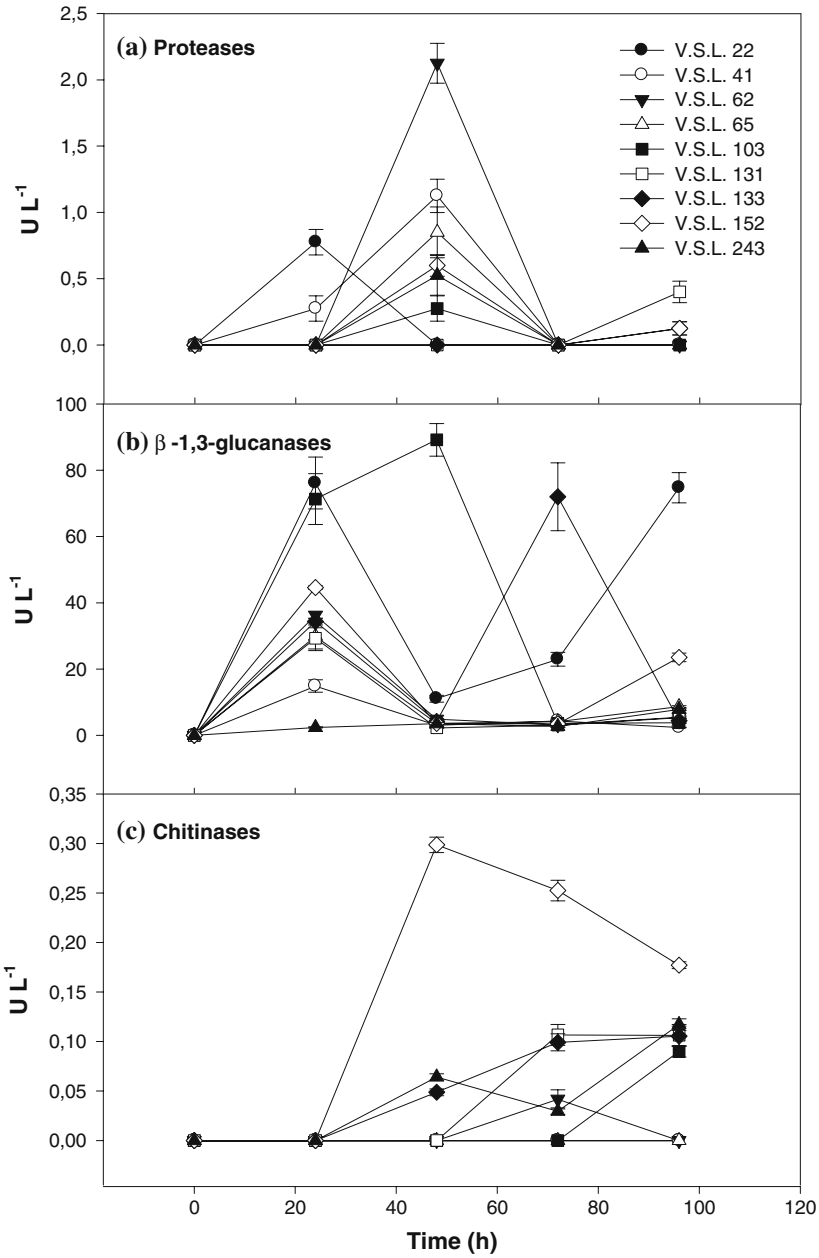

Figure 2. Extracellular enzymes produced by *Trichoderma longibrachiatum* strains cultured in liquid media. (**a**) Proteases. (**b**) β-1,3-glucanases. (**c**) Chitinases. Values represent mean ± SD of four replicates.

isolated, nine were selected because of their ability to fully inhibit growth of *Th. paradoxa* on PDA through the production of diffusible metabolites by the *Trichoderma* isolates. The inhibition of *Th. paradoxa* by diffusible metabolites produced by *T. longibrachiatum* is similar to the inhibition reported for growth of *Phytophthora erythroseptica* by *T. virens* (DAR 74290) [33]. Sreenivasaprasad and Manibhushanrao [34] demonstrated a moderate antibiotic effect of one isolate of *T. longibrachiatum* against *Rhizoctonia solani* and *Pythium aphanidermatum* but none against *Sclerotium rolfsii* whereas a second isolate had no antibiotic effect against any of these pathogens.

Mycoparasitism of pathogenic fungi by *Trichoderma* species is proposed as a mechanism of biocontrol and involves enzymes that degrade cell wall constituents (CWDEs) [35]. In order to test this premise, *T. longibrachiatum* strains were dual cultured with *Th. paradoxa*. In these cultures, contact between the hyphae of *T. longibrachiatum* and the plant pathogen was achieved at different culture times (between 38 and 52 h) according to the tested antagonistic strain (Table 2). After the establishment of physical contact, all the studied strains of *T. longibrachiatum* were able to grow over, to sporulate on, and completely to inhibit the mycelial growth of *Th. paradoxa*. Similar results have been found for other *Trichoderma*/pathogen combinations, e.g., *T. harzianum* against *Rhizoctonia solani* [10], *Crinipellis perniciosa* [36], and *Sclerotium rolfsii* [32], *T. harzianum*, *T. atroviride* and *T. longibrachiatum* against *R. cerealis* [37]. In their study of *T. harzianum* against *R. solani*, Benhamou and Chet [10] observed the first contact between the two fungi within 2 days of dual culture and a complete inhibition of *R. solani* immediately after this contact. These workers suggested that the antagonistic activity of *T. harzianum* was not due to the diffusion of toxic metabolites.

Scanning electron microscope studies showed that all the isolates of *T. longibrachiatum* parasitized aleurioconidial chains of *Th. paradoxa*, causing their loss of turgor, collapse and total disintegration. To our knowledge this is the first report of the direct parasitism of fungal spores of phytopathogens by species of *Trichoderma*. In this context, Davanlou et al. [38] demonstrated the parasitism of macroconidia and endoconidial chlamydospores of *Fusarium culmorum* by *Pythium oligandrum*.

It has been shown that *Trichoderma* species attack the host hyphae, forming coils around them [11, 34, 39]. We did not observe coiling of hyphae of *T. longibrachiatum* around hyphae of *Th. paradoxa*. Sreenivasaprasad and Manibhushanrao [34] observed hyphae of *T. longibrachiatum* to coil around hyphae of *Rhizoctonia solani* but not hyphae of *Pythium aphanidermatum*. We observed the antagonistic fungi growing closely along the hyphae of *Th. paradoxa* (Figure 1d). Although penetration and holes were not observed on the hyphal cells, the loss of turgor pressure, collapse and total desintegration of the phytopathogen hyphae were noticed routinely (Figure 1 e, f). These observations are consistent with observations by Benhamou and Chet [10, 11]. Sreenivasaprasad and Manibhushanrao [34] reported the formation of 'haustoria' like short branches in hyphae of *T. longibrachiatum* that were adjacent to hyphae of *P. aphanidermatum*, which became vacuolated and collapsed. We observed similar structures in our study (Figure 1a, b).

Extracellular enzymes produced by our *T. longibrachiatum* isolates were examined in liquid cultures. It was established that most of these strains produced the three main hydrolytic enzymes that are involved in fungal cell wall degradation (CWDEs). The highest activities of protease, β−1,3-glucanase and chitinase were detected after 48 h of culture, respectively for V.S.L. 62 (2.1 ± 0.15 U L^{-1}), V.S.L. 103 (89.2 ± 4.90 U L^{-1}) and V.S.L. 152 (0.3 ± 0.008 U L^{-1}) (Figure 2). This protease activity was substantially lower than that produced by *T. harzianum* T39 (58 U L^{-1}) and *T. harzianum* NCIM1185 (54 U L^{-1}) after 5 days of culture [26]. Equally, the chitinase activity was lower than that produced by *T. harzianum* NCIM1185 (197 U L^{-1}) after 6 days of incubation [25]. On the the other hand, β−1,3-glucanase activity was higher than that produced by *T. harzianum* T39 (1.1 U L^{-1}) after 5 days and *T. harzianum* NCIM1185 (1.48 U L^{-1}) after 4 days of culture [26].

Production of CWDEs by *Trichoderma* species depends on various cultural and physicochemical conditions such as the carbon and nitrogen sources, inductors, pH, temperature, time of culture, age of the inoculum and agitation [25, 40, 41]. Furthermore, high concentrations of glucose and ammonium are known to repress the biosynthesis of these extracellular enzymes [42, 43]. Additional experiments will be necesary to determine the adequate cultural and physicochemical conditions to optimize the production of CWDEs for each strain of *T. longibrachiatum*.

The results presented here show that the mycoparasitism of *T. longibrachiatum* on *Th. paradoxa* is made through the production of extracellular enzymes that degrade cell wall constituents of the pathogen, and that diffusible, nonvolatile metabolites are also involved in the antagonism of *Th. paradoxa* by *T. longibrachiatum*.

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